CRYOPRESERVATION OF HUMAN WHOLE BLOOD AND CHECKING THE VIABILITY OF MONONUCLEAR CELLS

A THESIS REQUIRED FOR THE PARTIAL FULLFILMENT FOR THE DEGREE OF

Bachelor of Technology

Biomedical Engineering

by

Stitiprajna Rout

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Under the tutelage of

Prof Kishna Pramanik

DEPARTMENT OF BIOTECH AND MEDICAL ENGINEERING NATIONAL INSTITUTE OF TECHNOLOGY

Prof. Krishna Pramanik
Department of biotech and
Medical Engineering
National Institute of Technology
Rourkela 769008

Phone no 0661- 2462283
Mobile no 9437191611
Email- kpr@nitrkl.ac.in

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the project entitled "**Cryopreservation of human whole blood and checking the viability of mononuclear cells**" is a legitimate piece of work carried under my supervision as a part of work required for the completion of Bachelor's degree in BIOMEDICAL Engineering in National Institute of Technology and to the best of my knowledge this thesis or any part of this thesis hasn't been submitted to any University/Institute/College to the best of my knowledge for all intents and purposes

Prof Krishna Pramanik

Dept of Biotech and

Medical Engineering

(signature)

ACKNOWLEDGEMENT

I offer my sincere heartfelt gratitude to Prof Krishna Pramanik without her the project for the life of me couldn't have been possible. During the course of project she was my guide, mentor, philosopher all rolled into one. I am in particular thankful to her for giving me the autonomy to pick the project and autonomy on various other things at the same time she controlled me just like the way a kite is controlled by the master after it is given the freedom to soar high to touch the clouds. By making my foray into cryopreservation I knew I would be joining the ranks of the people who are taking small steps for a dream that would culminate into the proverbial giant leap for the mankind. That means there is a need of certain amount leeway and latitude to be provided which Prof Pramanik was generous enough to provide. She was a constant stream of inspiration for me. During the course of project she handed me down some life lessons which stems from the fact that she is a repository of knowledge, wisdom experience and patience. She despite her hectic schedule inculcated in me the correct attitude and approach to research which can be applied to various other research fields back and forth in time

I would also like thank (Ph.D Scholar) Akalabya Bissoyi for schooling me on everything I asked to him be it complex or wishy-washy and most importantly he schooled me on things that I needed to learn but was dumb enough even to figure it out. Rajdeep Kaur, (Ph.D Scholar) for keeping me chipper and enthusiastic and also for donating blood.

I thank Mechanical Dept for letting me use liquid nitrogen and SEM

Finally I thank my family frankly I don't think without them I could have come this far in life and all those who have played whatsoever part in the project.

ABSTRACT

Now a days, cryopreservation of whole blood & whole blood derived mononuclear cells (MNC's) has emerged as a promising technique for the healing of the subjects going through various blood related ailments. The supremacy of this technology are amazing cell viability, the cell traits remain intact i.e. the retention of the structural and functional intactness of the super freezed cells after decades of preservation However, there are a host of challenges in cryopreservation while preserving the cells due to different levels of attached intricacies that involves achieving cell viability, cell function, cell proliferation and differentiation. The present work investigated the outputs using some selected cryoprotectant on post thawing viability mononuclear cells (MNCs) derived from peripheral whole blood during cryopreservation process along with just preserving peripheral whole blood. The extracellular cryoprotectant such as Dextran and HES with a concentration of 10% w/v and intracellular cryoprotectant such as glycerol and DMSO with a concentration of 10% v/v were used. Post thaw cultured samples were analyzed for cell viability by trypan blue membrane exclusion assay. Samples consisting of hydroxy ethyl starch (HES) were found to be the most effective cryoprotectant achieving average cell viability of 84 %. Whereas only 63% cell viability was obtained using Dextran as cryoprotectant. SEM and phase contrast microscopy confirm the normal cell morphology of the post thaw cultured whole blood and mononuclear cells respectively

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CHAPTER 1

INTRODUCTION

- **Motivation**
- **Introduction**
- **Literature review**

1.1 MOTIVATION

After I was assigned under Prof K.Pramanik. On a sultry afternoon one of my friend who was also assigned under her informed me that she wanted to meet me at her leisure time to discuss in detail about our project.She being the Dean (Student Affairs) is very busy but she is always there for her students.I at a convenient time went to her chamber as I was approaching her chamber I felt a tremor of shyness because (a) I was the apprentice of one of the best Profs in India that there is in Bio Enginering (b) I being a rookie didn't expect to stand on her expectation and meet the challenge.I was self effaced about the whole thing.Finally when I entered her chamber we had a sit-down talk she gave a whole lot of ideas but deep down she didn't want me to go along with any of those ideas unless I really wanted to proceed with it.She knew if I don't like my project my input would be low and hence output would be botched up and I will see the resarch as a chore as a routine ,as a punishment.Therefore she urged me to find out what I am interested in and she gave me some time off to straighten my priorities.She told me if I came up with any idea that is possible under the umbrella of NIT Rourkela I have her complete backing.She asked me to read different resarch papers talk to my friends and I still don't figure it out she is always there to help me.

Now on one morning after doing pranayama I was sitting all by myself in my room when somebody played a song that I used to hear in my freshman year.Boom a flash back comes to me and answers the impending question.During my freshman year we didn't have much clubs as we have now, mostly we had social clubs but they were contagiously infective to the freshmans who wanted to revolutionize the lives of people.I because of involvement with social clubs receive a phone from a person saying that he urgently needs a unit of O(-) blood group.I rush down immediately to my wardens office and tell him that Sir a certain somebody has called requesting a unit of O(-) blood group.Our warden spear headed the blood donation activity and we were just figure heads.He opens his computer and out comes a hardcopy about 8-9 pages.He staples it and hands it over to me I had to wink my eyes twice as I go through each paper because I couldn't find any O(-) finally after going through the sheaf I found two freshmans having it.With much difficulty I tried to glean their room numbers and phone numbers.I knocked at the first guys room when I asked him to donate his blood he was scared to no end.I did a text book on him as I was instructed by our warden Prof KR Patel but to no avail .He was a cop out so blood donation was an absolute no no for him.The second guy was a pushover initially although apprehensive I dispelled his

doubt.Despite that on our way to IGH hospital he was hyperventillating in the auto and was rattled after we landed at IGH and after he donated blood he received a pint of milk and two eggs from the hospital .He told me that he is slightly feeling dizzy.I told him that its all pshychological and it will go away very quickly.Meanwhile I was so elated at the sucessful completion of my first social assignment that all food items I could find from the small convenience store inside the hospital's premises I brought it for that boy starting from cashewnuts to peanuts,to pineapple juice,to cold drink ,to ice tea,to chips.I spent around 200 bucks on that.The boy had to carry it with his arms crossed arms and was awfully chipper.He looked like a boy from some banana republic who has been offerd a candy.that night I understood the importance of O(-) blood group and that was all the motivationI needed to get started with this project.

1.2 INTRODUCTION

1.2a Cryobiology

They say (scientist) that a single cell is many order of magnitude complex than a space ship what to speak of tissues and organ. Cryobiology is the science that studies the impact of low temperatures on biological system. The motive, agenda behind cryobiology is to preserve cells, tissues, organs by arresting biological time without any changes in their features ranging from genetic to morphological to functional etc**. Cryopreservation** refers to the storage of living biological system at very low temperatures (-196^{o}c) in (liquid nitrogen) for duration of time such that it can be reinstated and retrieved to the same living state as before. It's the singular, widely applied approach to long term suppression of biological function. Restoration of structure and function can be done after decades of liquid nitrogen storage. **Cryopreservation** doesn't have any peers it is in a class all by itself

1.2b Process

- Relatively simple
    - Addition of cryoprotective agents into cells/tissue
    - Cooling process-controlled rate
    - Storage at low tempratures-196^0 C
    - Warming process-rapid
    - Removal of cryoprotective agents from cells/tissues-process dependent

Each parameter is important as they are intertwined and may directly or indirectly interfere to dictate the outcome

1.2c Some popular methods of preservation

(a) Maintenance in tissue culturing by serial sub –culturing
(b) Storage at refrigeration temperatures (-4 to -20°C)
(c) Freeze drying
(d) Vitrification
(e) Cryopreservation

(a)Maintenance in tissue culturing by serial sub culturing

The major disadvantage of this method is that cell can only survive for short spans that is cells can't be kept for longer periods of time moreover there is always a risk of running into infection through bacterial, viral and other sources

(b)Storage at refrigeration

The same problem persists in this mode of preservation also. After a while cells start losing their viability and this method can't be effective for all cell lines

(c) Freeze-drying

{1,2}(Adams 1996; Gheorghiu, Lagranderie et al. 1996) It is a preservation process that uses freezing, drying vacuum, heating and low pressure. The substance to be freeze dried is first freezed and then the ambient pressure is decreased and heating is done as a result the frozen water changes from solid to gas by (sublimation).The process of freeze-drying process is also called lyophilisation or cryodessication. After freezing the next thing is to do sublimation on process on the sample. It is necessary to decrease the partial pressure of water beyond triple point to ascertain the straight change of ice into vapor and to stop melting of the sample. Vacuum is necessary for Promoting sublimation and for taking care that the air doesn't escape the system {3}(Rey 1999)

NB{4} (Cammack and Adams 1985)Vaccines that are prepared by this process should need any inert substance or a vehicle for it to minimize the damage when uncovered to humid conditions

Applications of freeze drying involve

- Astronauts using freeze dried foods in space
- Pharmaceutical company use it to increase the shelf life of product as vaccine
- Food and Biotechnology etc

Vitrification

{4}(Varshneya 1994)It is the transition of a substance to glass by very fast cooling. The liquid has to passing through ($\mathbf{T_g}$). ($\mathbf{T_g}$).is the glass transition temperature for non-crystalline substance below which the substance acts as glass (hard, brittle) and above which it acts like rubber(can bend).

Vitrification in nature

On account of processes like vitrification life thrives in extremely cold and frigid conditions like in the case of arctic frogs. Vitrification happens with water if there is some cryoprotectant which inhibits the formation of ice crystals. The liver of frog releases loads of cryoprotectant in form of glucose{5}(Storey 1990)which permeates the cell because of typical insulin released which prepares for the additional uptake of insulin and hence ice crystal formation is stopped{6} when the winter thaws the glucose is removed and is stored for metabolic activity

NB The major flaw while using vitrification for preservation is the toxicity caused by higher conc. of cryoprotectants{7}(Lawson, Ahmad et al. 2011)

**For the source for text given in introduction section see page 40

1.2d Why cryopreservation of human whole blood

Let me preface by saying one of the major reasons of cryopreservation is to preserve a rare blood group for an incredible length of time for emergency purposes about which I gave an anecdote in 1.1

{8}(Schinndler ,Asmus et al).The second major reason was the increasing use of blood assay for pyrogenic testing as well as immunogentic testing because of the ready made physical environment,hassel free operation ,reduced concotion etc.This nudged me for cryopreserving leuckocyte having the epithet the police force of our body to find out their viability

Hematopoietic stem cells can be derived from peripheral blood and recent development in medical science shows that hematopetic stem cells when injected into ischemic lesioned area there was a significant improvement in that area.{9}As well as Hematopoietic stem cells can reduce post ischemic inflammation (Schwarting, Litwak et al. 2008) .

Erythrocyte disorders.

It can be stated as the disorder which are detrimental to RBC , reduces its production and causes destruction of RBC.Anemia is one of the major disorder of RBC resulting in decrease in O_2 carrying capacity due to decrease in RBC levels or less haemoglobin {10}(Gasche, Berstad et al. 2007)

Table 1 showing anemia grading system{11}(Bridges, Bridges et al. 2007)

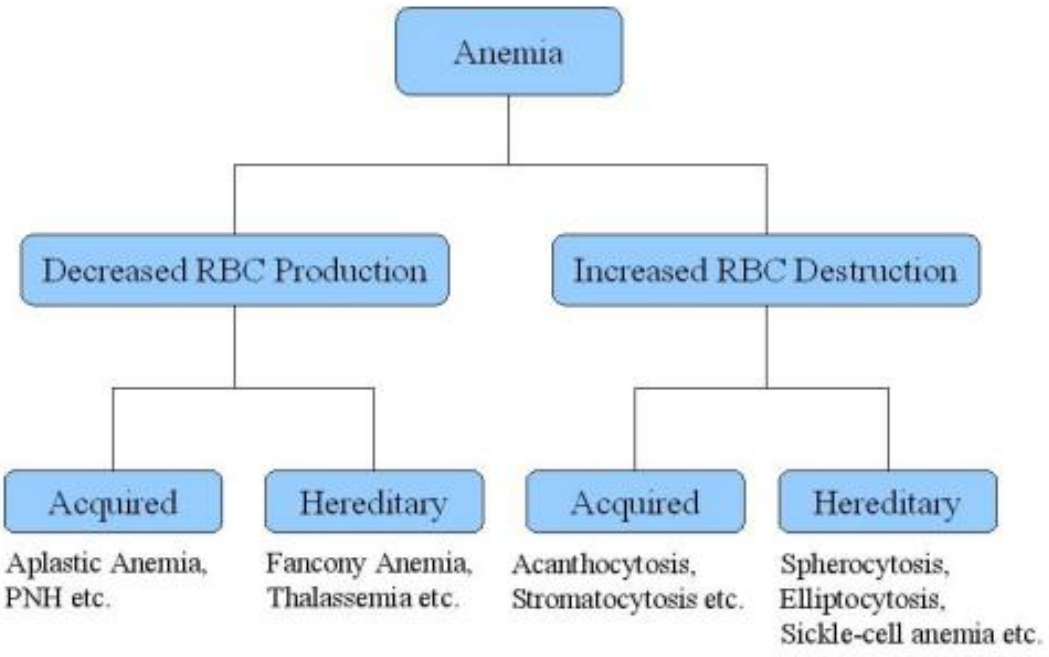
Severity	WHO	National Cancer Institute
grade	.>=11 g/dl	wnl
grade	9.5-10.9 g/dl	10.0 g/dl
grade	8.0-9.4 g/dl	8.0-10 g/dl
grade	6.5-7.9 g/dl	6.5-7.9g/dl
grade	<6.5 g/dl	,6.5g/dl

Wnl (within normal limits) 12-16g/dl for female and 14-18g/dl for man

Some more erythrocyte disorders are

- Sickle-cell disease
- Thalassemia
- Spherocytosis
- Pernicious anemia
- Aplastic anemia
- Pure red cell aplasia
- Hemolysis
- Hereditary spherocytosis

Because of so many RBC disorders it makes sense to preserve whole blood which is almost 45%RBC



1.3 LITERATURE REVIEW

1.3a Advantages of cryopreservation

(a) It needs low concentration of cryoprotectants
(b) High cell viability
(c) Cell maintain their characteristics
(d) Cells/tissues can be stored for a very long period of time

1.3b successfully applied to

- Semen {12}(Watson 1995)
- Blood
  - Special cells for transfusion
  - Stem Cells.
  - Umbilical Cord
- Tissue samples like Tumors and Histological cross sections
- Eggs (oocyte)
- Embryos that are 2, 4 or 8 cells when frozen {13} (Trounson and Mohr 1983)
- Ovarian Tissue
- Plant seeds or shoots may be cryopreserved for conservation purposes.
- Cornea
- Skin
- Pancreatic Tissues

Cryopreservation although has been successfully applied to above mentioned things but when it comes to heart liver, kidney ,organs it still remains a herculean mountain to climb

NB In most cases of cryopreservation there is a lack of systemic protocol. Most of the time it becomes a judgmental call by the cryopreserver after factoring in the complexities. Contamination from donor derived adventitious agent is also an issue

1.3c Variables that regulate the pulse of cryopreservation

(a) CPA- Cryoprotective Agents

They can be

(a) Intracellular

Low molecular weight

Can permeate cell

E.g. DMSO, Methanol,Glycerol

(b)Extracellular

High molecular weight

Don't permeate cell

E.g. poly vinyl pyrolidine, sucrose

(342 Da), hydroxy ethyl starch

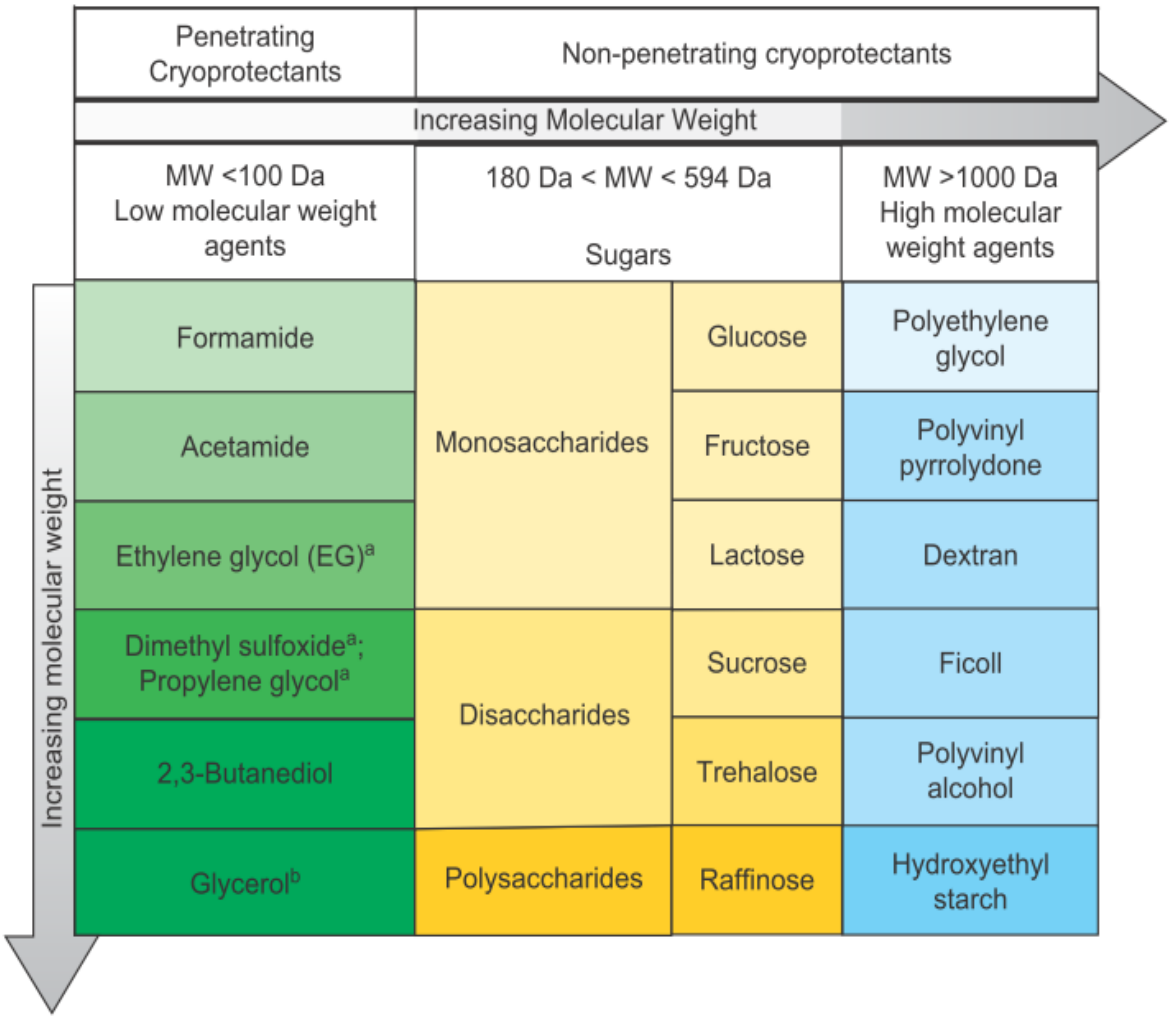
Working process of Cryoprotective Agent

- They decrease the fraction of solution frozen at a given time
- Minimize intracellular ice formation
- Induce extracellular glass formation
- Suppress high salt concentration

NB The choice of cryoprotectants is dependent upon the type of cell to be preserved

E.g. Example glycerol preferred over DMSO for toxicity reasons

TABLE 2 SHOWING VARIOUS CROPROTECTANTS IN THE INCREASING ORDER OFMOLECULAR WEIGHT



NOTE THE CORRELATION BETWEEN MOLECULAR WEIGHT AND PENETRATING CAPACITY

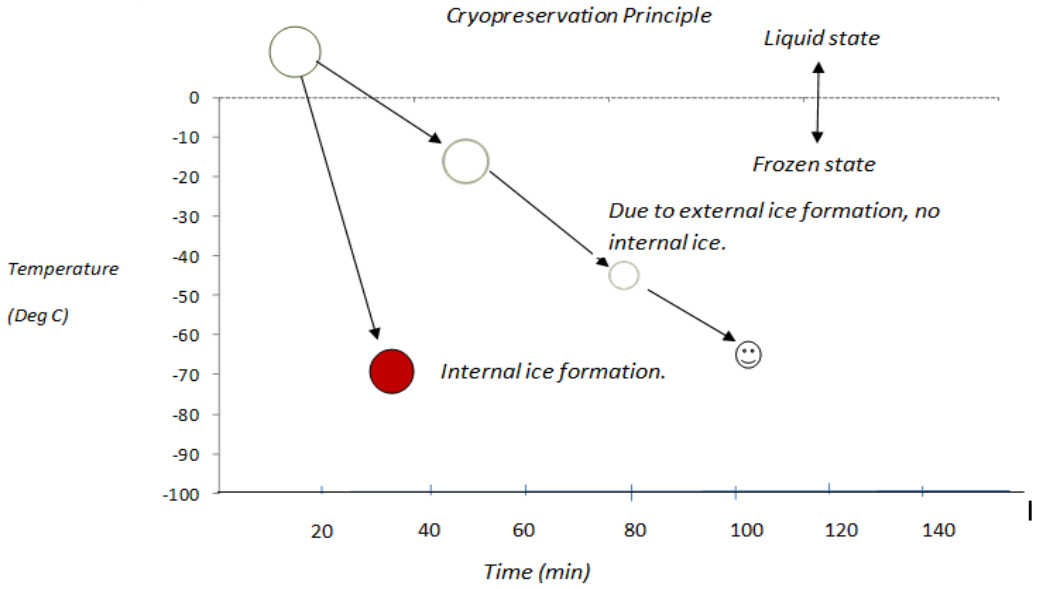
(b)Rate of cooling {14,15}(Sutton 1991; Morris)

Cooling rate is supposed to have a great impact on cell survival and viability. Control rate freezing before durable term storage enhances viability for the cells. Regulated constant cooling rates are successful for a plethora of freezing purposes

Rapid rate of cooling causes formation of intracellular ice crystals which are detrimental to cells causing mechanical injuries. In this scenario the role of intracellular cryoprotective agent as discussed earlier becomes very significant

While in slow rate of cooling there is formation of osmotic difference due to different solution condition and generation of extracellular ice, these factors may harm the cells by diffusion of water outside of cells and cells may shrink and lead to osmotic dehydration. A slow cooling causes freezing of the extracellular fluid whereas intracellular fluid remains unfrozen for a while. This results in an increase in the concentration of salt in extracellular solution which leads to cellular dehydration and osmotic flow through cell membrane. In this case the role of extracellular cryoprotectants becomes important

Figure 1 showing rapid and slow cooling effects {15}(Day 2007)



Schematic diagram that shows when cells are cooled rapidly intracellular ice formation happens and when slowly extracellular ice formation takes place

Therefore the key is to optimize the cooling process

Figure 2 shows cooling optimized cooling rate {16}(Gao and Critser 2000)

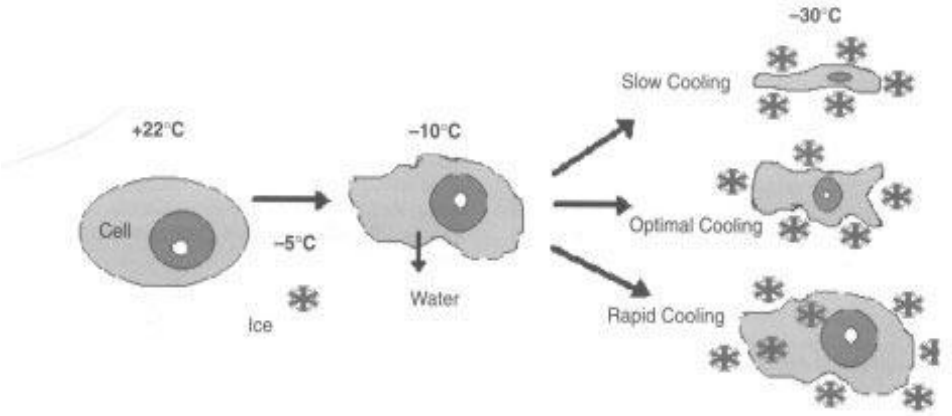


Figure 2 showing not optimized cooling

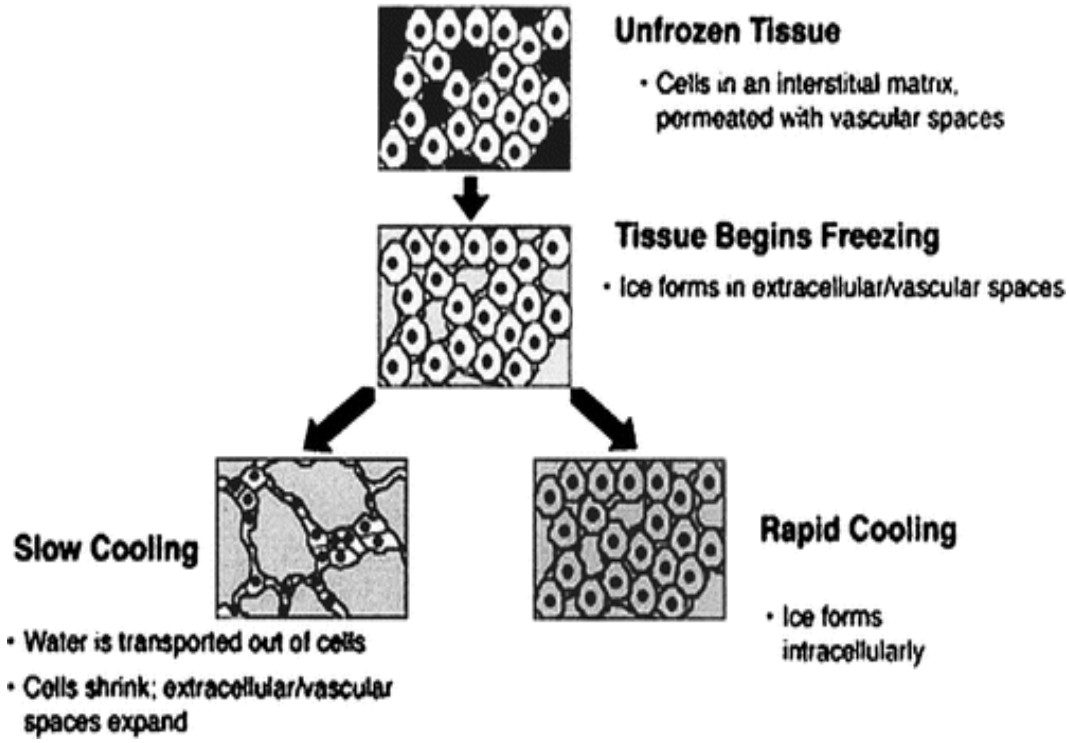
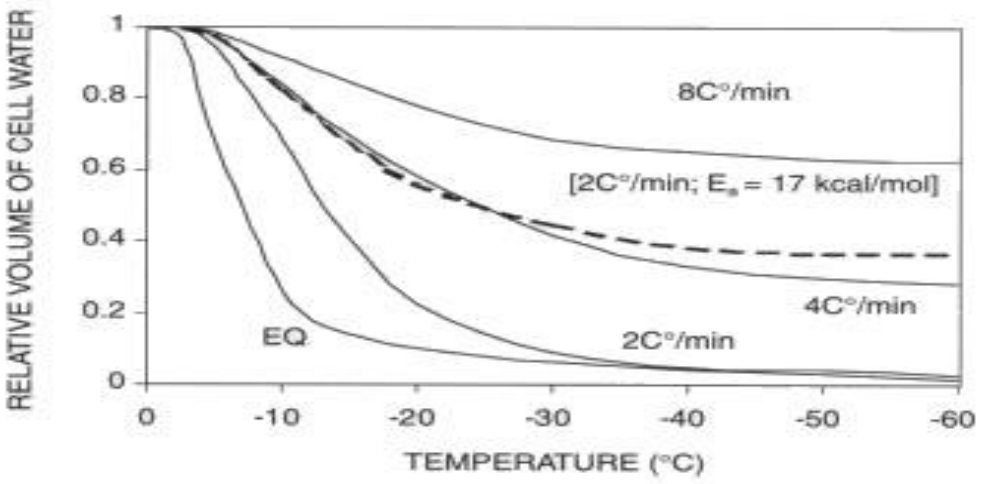


Figure 3

Showing the kinetics of (H_2O) elimination in rat's ovum cooled at 2 to 8C/min in 1 M dimethyl sulfoxide. The line EQ should be the intracellular water amount that the cell must have for correct chemical potential between intra- and extracellular ice, as a function of temperature. The other solid lines, marked2, 4, or 8C/min, were calculated assuming the activation energy (E_a) to be 14 kcal/mol) {16}(Mazur 1984)



SOME HISTORICAL FACTS

In 1964 Mazur.P found out that the way we regulate cooling actually decides the movement of water across cell membrane and hence intracellular freezing(Mazur 1963){17}

(C) Thawing

The warming re-warming rate if not controlled for cryopreserved substance in the vials can be deeply detrimental to the purpose of cryopreservation; can affect their mortality, morphology, activity etc. Re-warming if tinkered or fiddled with can adversely affect the outcome. Re-warming should be done rapidly otherwise small ice crystal would come together to form bigger ice crystals hence damaging cells The motive is to return cells to their original physiological condition This brings back the osmotic challenge as an gradient slowly begins to form across plasma membrane To get rid of this problem the cryoprotectant is diluted in a stepwise manner gradually at proper time intervals to maintain cell volume below the limit for damage

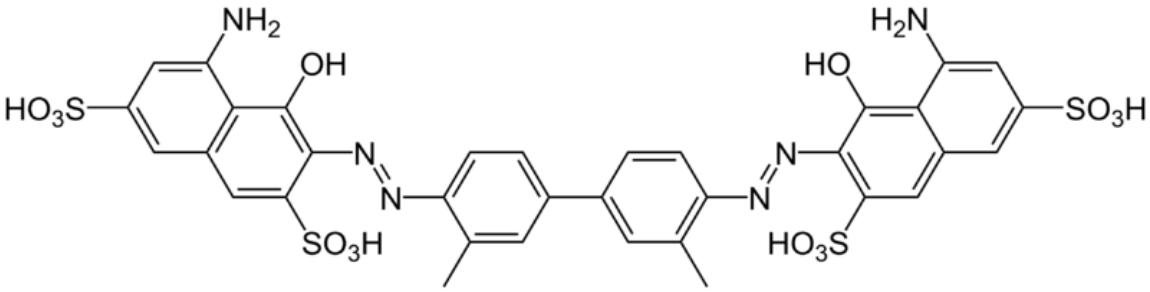
Protocol for thawing

The vials are taken out with the help of tweezers from the liquid nitrogen container. One should be careful and use customized gloves and glasses for this purpose

- Thawing is done maintain the water bath at 37°C and caution must be exercised to prevent cracking of vials
- Vials are then washed with 70% ethanol
- Vial should be opened properly avoiding contamination
- The contents then should be transformed into pre-warmed medium(Coriell 1979;){18}

(d)Post thaw culture Assays of different types such as trypan blue are used to check for cell viability. Trypan blue colors the dead cells blue and doesn't color the living cell. Since the cell membrane are of living cell are semi permeable and intact it doesn't allow trypan blue to penetrate through unlike dead cells

Figure 4: Trypan blue structure



1.3e{19}(Day 2007)Mathematical analysis of osmosis and diffusion in case cryopreservation

Cryoprotection in essence is the ministration of cells and tissues with cryoprotectants many times in high dozes which enables the passage of water through osmosis and solutes move by diffusion. Freezing means formation of ice and decrease of water & increase of solute hence establishes osmosis and diffusion .Membranes as they are selectively permeable react indifferent ways according to solute sometimes enabling it to diffuse sometimes not. There is on this basis equilibrium challenges

The flow is on account of pressure. The flow via membrane is

$$J_v = \mathrm{k}.P \quad ..1$$

k is the constant for membrane along with water factored in. P is the pressure difference j_v is the volumetric flux

When the force is due to osmotic pressure Π equation becomes

$$J_v = \mathrm{k}.\pi \ldots 2$$

In actual scenario both the above equation are combined to give

$$J_v = L_p(\mathrm{P}+\pi)$$

L_p is same as k as has same value in 1 an2 and is called hydraulic constant

When hydrostatic pressure term is zero and π can be estimated by conc by X with molar gas constant and the temp. If C_i and C_e are internal and external osmolarities respectively and A is the area of the membrane then

$$J_v = L_p.A.R.T.(C_i - C_e)..3$$

The solute flux

$$J_s=\omega_s.A.R.T(S_e - S_i)\ \ldots 4$$

ω_s is solute permeability

{20}(Kedem and Katchalsky 1958)Kadem and Katchalsky took into consideration that the solute & solvent use a common gateway to enter membrane and modified the 3 and 4th equation by using σ for the solute and solvent communication called reflection coeff.Now the new equation becomes

$$J_v = L_p.A.R.T.[(C_i - C_e) + C_i^P\text{-}C_e^P)]..5$$

$$J_s = \omega_s.A.R.T.[(S_e - S_i) + J_v(1-\sigma)c_s]..6$$

C is impermeating solute and C_P is permeating small c is conc

**For the source for text given in literature review section see page 40

CHAPTER 2

EXPERIMENTS

- **Cryopreservation of human whole blood**
- **Cooling curve**
- **Studying Human whole blood under SEM**
- **Isolation of MNC**
- **Cooling curves of MNC**
- **Trypan blue staining protocols**

EXPERIMENTS

2.1 Cyopreservation of human whole blood

Blood from healthy volunteer was taken to rule out any possibility of infection

Vials include the following cryoprotectants Five batches of blood was taken first without cryoprotectant and others with 10%v/v DMSO,glycerol 10%w/v dextran and HeS. After cooling vials were stored in liquid Nitrogen

Table3: Depicting cryoprotectants along with v/v ratio or w/vratio

1	----- --	No cryoprotectant
2	10%v/v	DMSO
3	10%v/v	Glycerol
4	10%w/v	Dextran
5	10%w/v	Hes

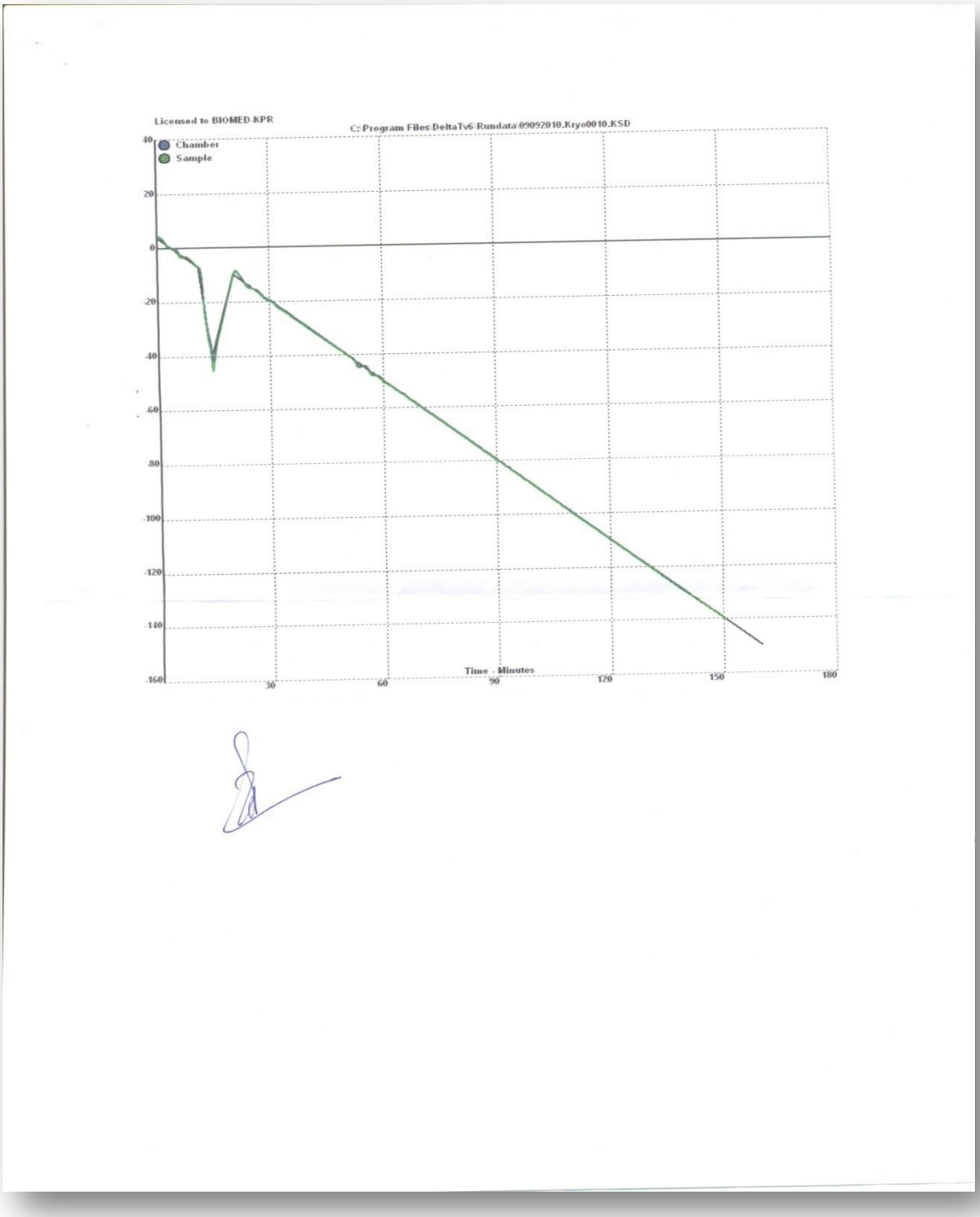
After that the controlle rate freezer was programmed as shown in the table

{21}(Day 2007)Cooling table 4

Step1	Initial temp 4^0C	------
Step2	4^0C to -7^0C	@-1^0C/min
Step3	-7^0C to -40^0C	@ -15 ^{0}C/min
Step4	-40^0C to -10^0C	@ 10 ^{0}C/min
Step5	-10 ^{0}C to -150 ^{0}C	@ -1 ^{0}C/

2.2

Graph 1 plotted from controlled rate freezer



2.3Protocols for studying Human whole blood under SEM

- Glass slides of diameter 19 mm are made using glass cutter and washed in an ultrasonic bath for 5 min.
- A 10 µl drop of post thawed MNCs (5 x 10^7 cells/ ml) was placed on a coverslip and 10 ul of poly-L lysine in PBS was added immediately before the cells had time to settle onto the glass, to give the final poly lysine concentration quoted in the results.
- After 5 min, the polymer-treated cells were fixed by addition of 10 µl of 2% glutaraldehyde in PBS for 2 min. The coverslips containing the fixed samples were placed in a vacuum desiccator and transferred into a beaker of PBS. They remained overnight in the PBS at 4°C.
- Cell dried using a series of graded alcohols from 30% to 100% ethanol .Dried samples coverslips were sputtercoated with platinum and observed with a Jeol JSM 6700F field emission scanning electron microscope operated at 5 kV.

Figure 5 whole blood as seen under SEM

The RBC's appear very robust with Hes as cryoprotectant

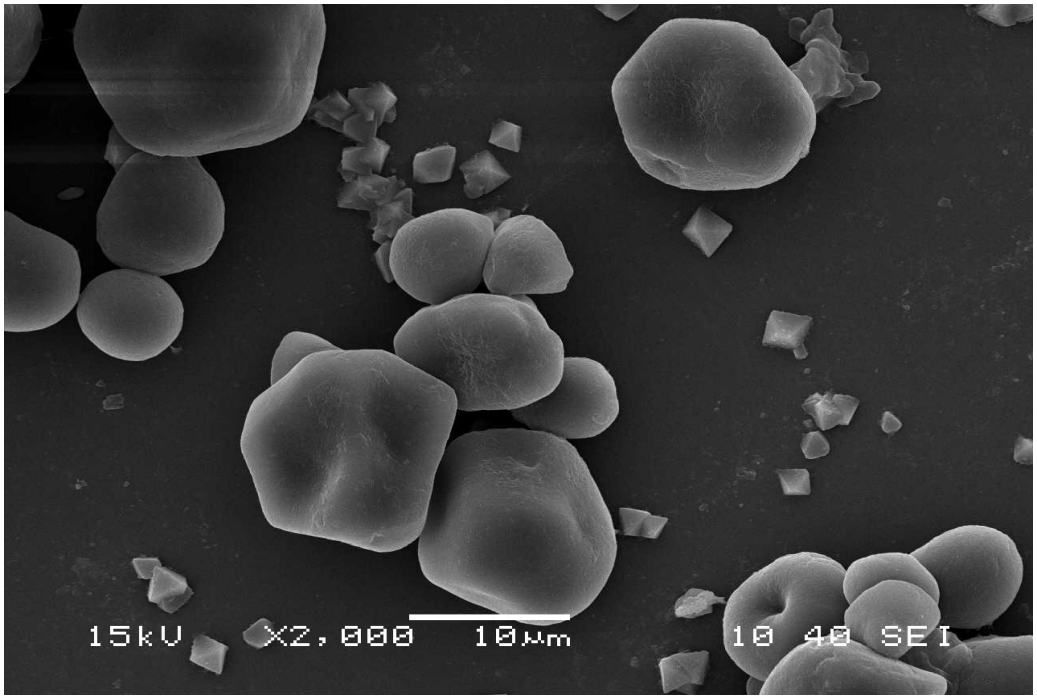
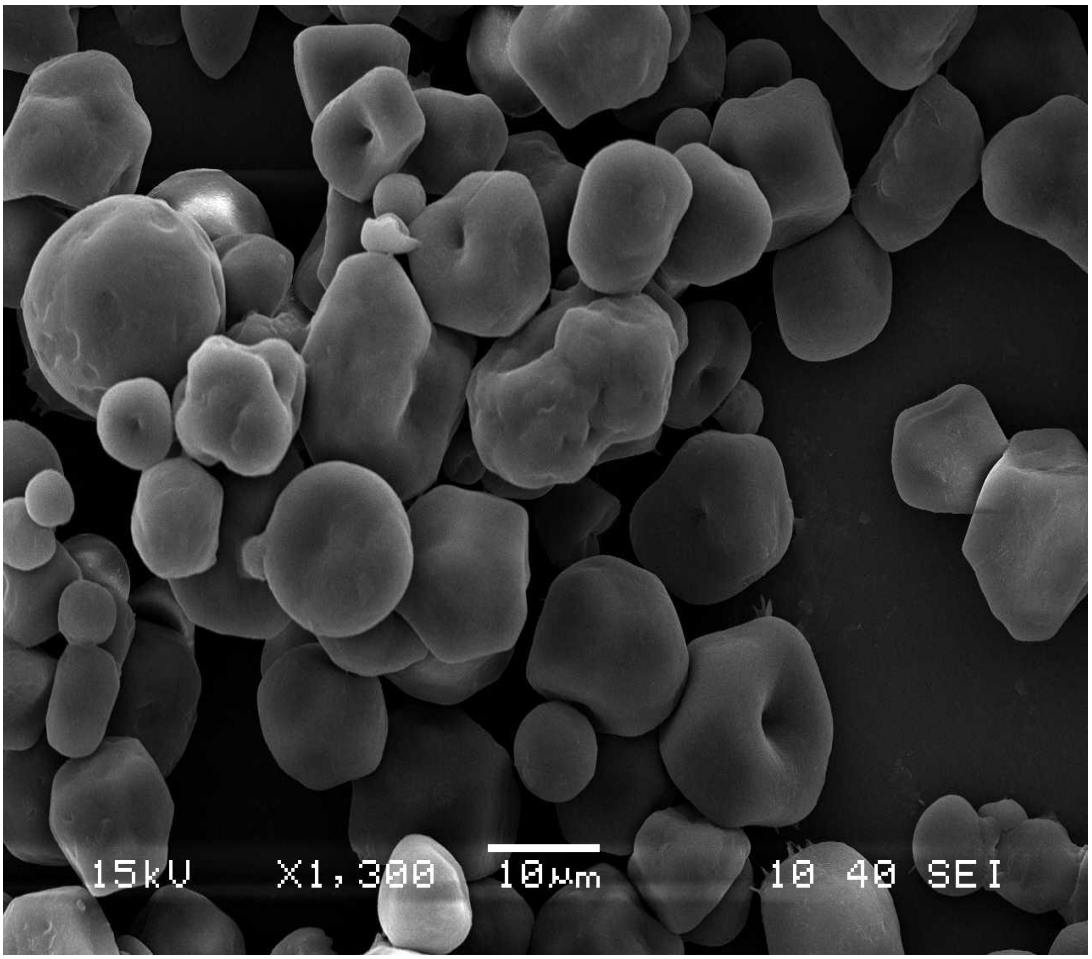


Figure 6 another SEM image mostly the cells are RBC as it ranges in millions in human beings compared to MNC's which is in few thousand and platelets which is tough higher than MNC's not significant portion of blood cell population when they are compared to RBC



{22}(Day 2007)

2.4 MNC ISOLATION

Human whole blood

Isolation of MNC (By density gradient centrifugation using ficoll hysep)

Cell counting

Primary culture

ISOLATION

Ficoll +blood and DPBS(1:2)

Centrifuge at 2000rpm for 30 mint

Aspirate the cloudy layer

Wash it twice with DPBS at 1500 rpm

Count total num of cell and transfer it in

Culture plate along with suitable media

{23} (Boyum 1968)

Centrifuge separation of MNC protocols

The cells are diluted up to 2 to 4x buffer volume. The greater the dilution the better the MNC yield

A conical tube of 50 ml is taken on that 15ml of ficoll paque is added and 35ml of ml of the diluted cells are poured gently

Conical tube is put in a centrifuge for half an hour @400Xg

The upper layer is aspirated allowing the PBMC to be untouched. This should be done very delicately

Buffer sol is now poured (PBS, EDTA)and centrifuged @ a temp of 20°c FOR 10 MINS @300Xg.The supernatant liquid is poured over

In order to remove platelets the cell pellet is suspended in % 50ml of buffer and is centrifuged @ 200xg

This step is repeated once more

The cell pellet is suspended in desired quantity of buffer for magnetic labeling

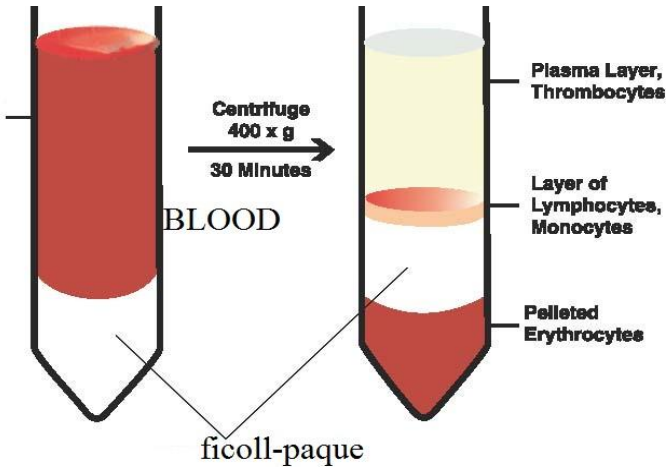


Figure 7 Isolation of mononuclear cells Schematic picture

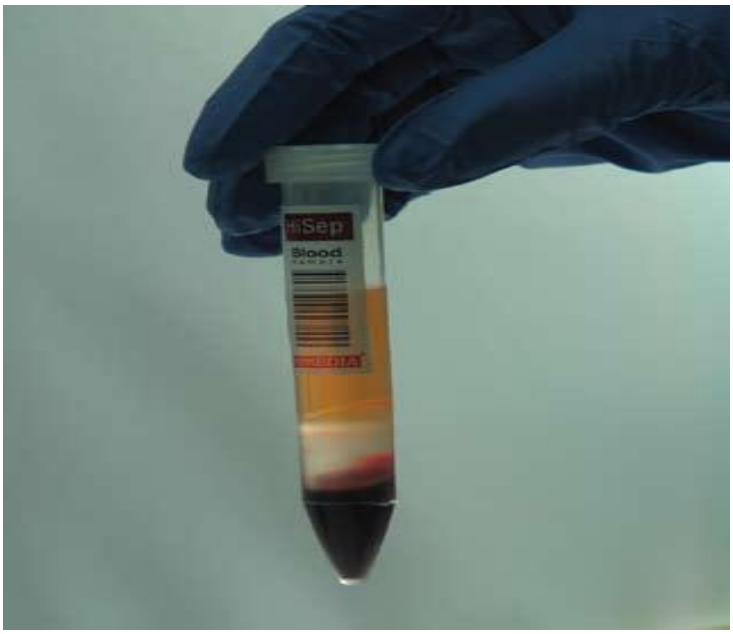


Figure 8 shows MNC as seen in a real exp observation

Picture taken from a online gallery source ofAssociate Prof. Dr. Tunku Kamarul Zaman Bin Tunku Zainol Abidin

{24}(Day 2007)

2.5 Cooling plots of MNC

Staring temprature-4°C

Step-1. Initial rate of cooling-(-1/min)

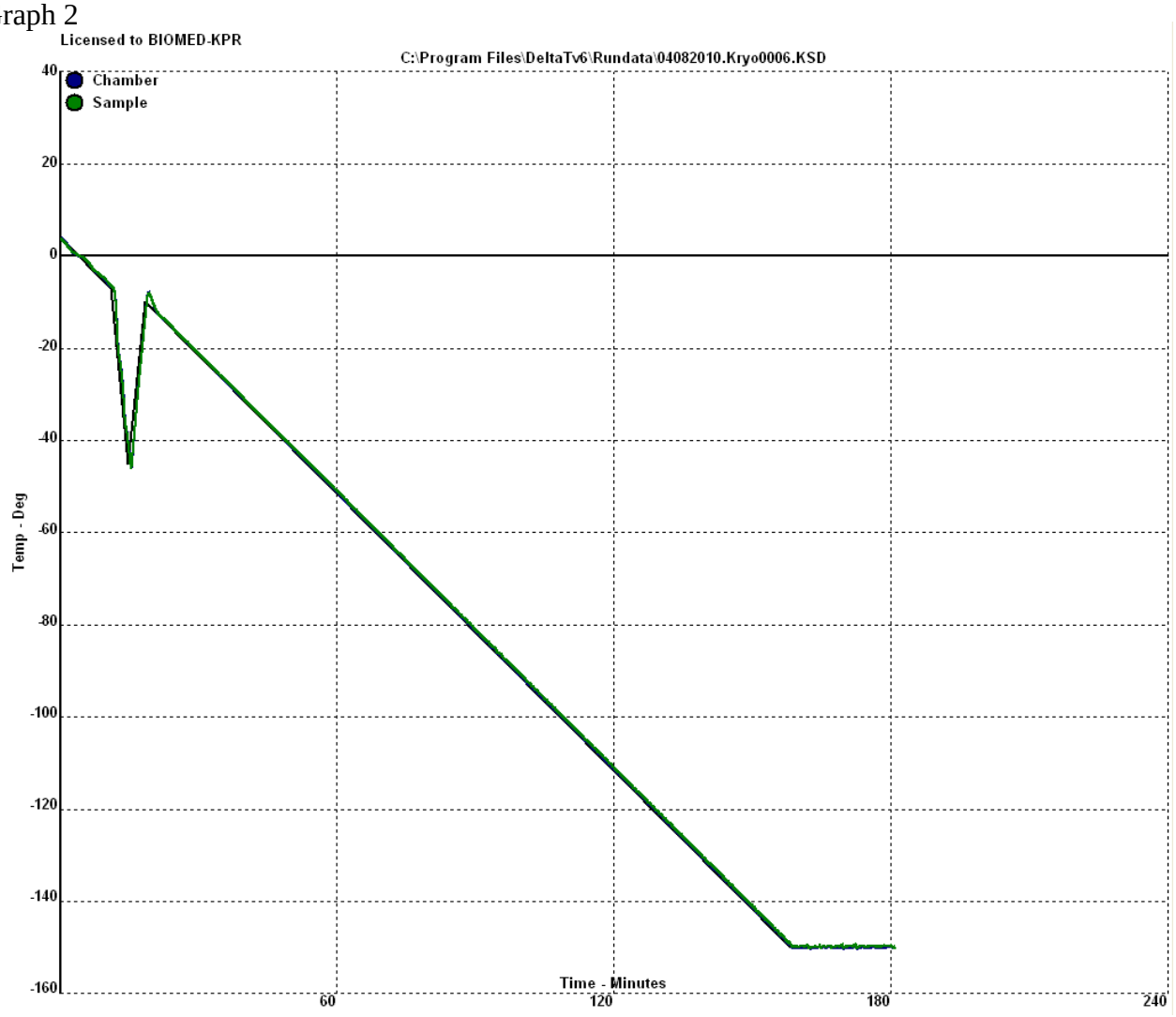
Step-2.-15/min to 40°C

Step-3. 10°C/min to -10°C

Step-4. -1/min to -180

Step-5. plug in liquid nitrogen Cooling plot1

Graph 2



Cooling Plot of MNC no2

Staring temprature-4°C

Step-1. Initial rate of cooling-(-1/min)

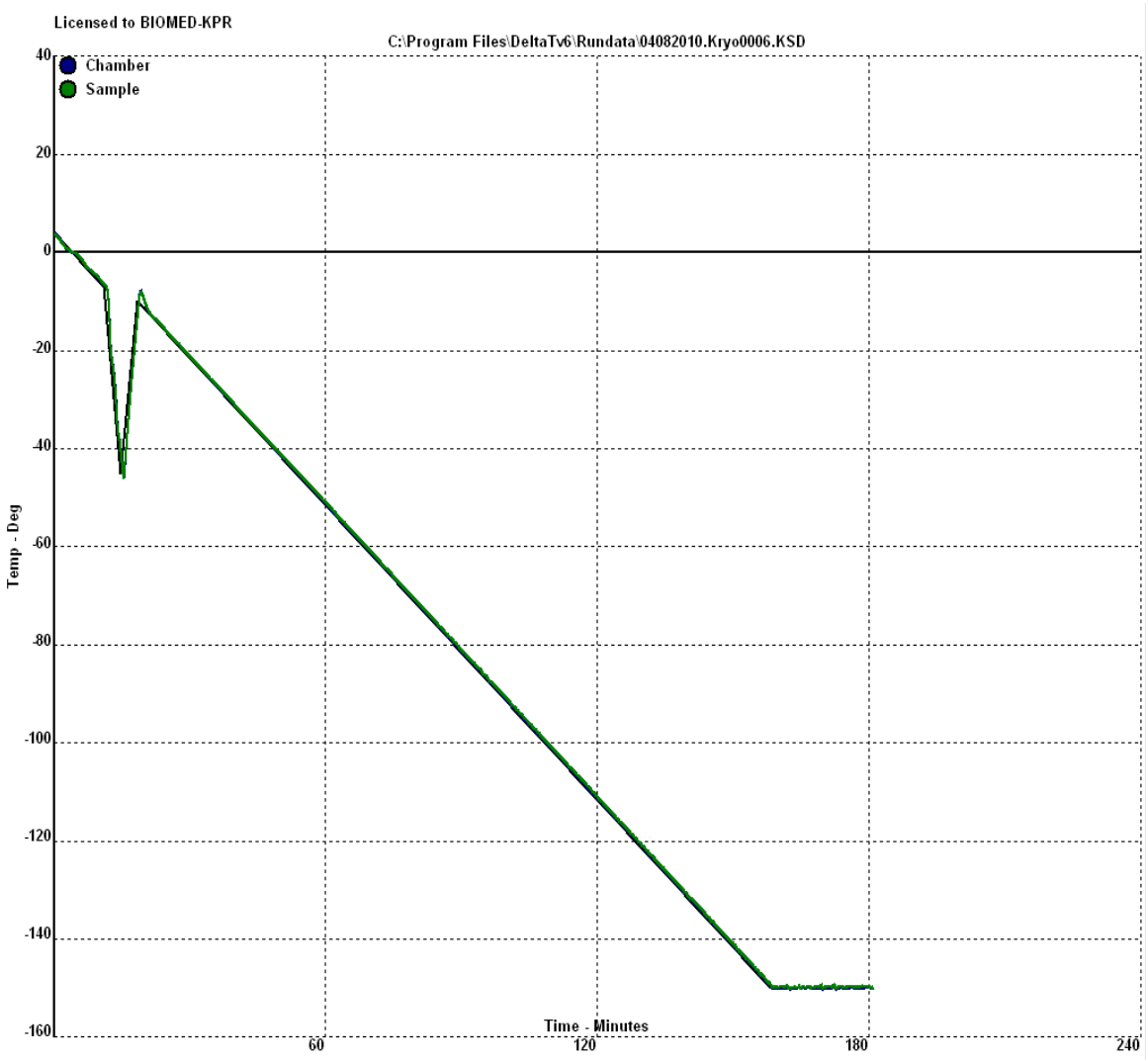
Step-2.-15/min to 40°C

Step-3. 10°C/min to -10°C

Step-4. -1/min to -180

Step-5. plug in liquid nitrogen

Graph 3



2.6TRYPAN BLUE STAINING PROTOCOLS{25})(Shapiro and Leif 2003)

Material needed

PBS

Serum free complete medium

O.4% trypan blue should be kept in dark container

(a) The cell suspension which is checked for viability is centrifuged@100Xg &the supernatant is rejected. Aliquot should have enough amount of cell for counting after dilution in PBS and trypan blue

(b) The cell pellet is suspended in PBS or non-serum free media, Serum protein stain along with trypan blue and the results would be in accurate. Therefore non serum environment is preferred

(c) O.4% of trypan blue is mixed with 1 part cell suspension. The mix is allowed to incubate at room temp

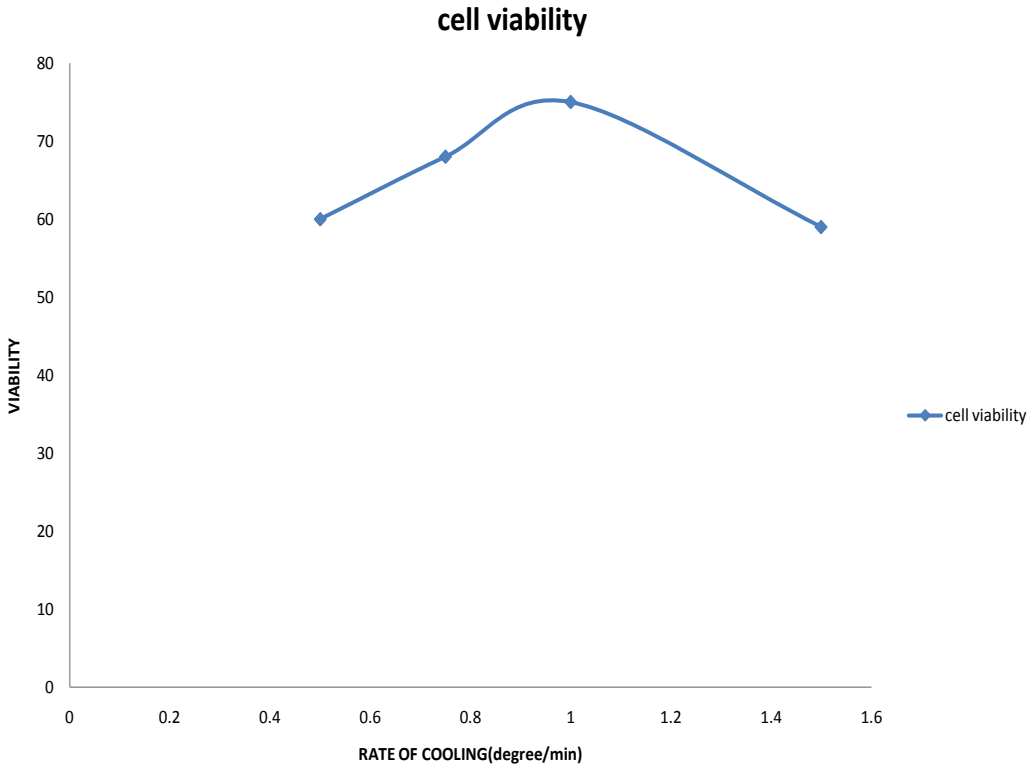
NB cells must be counted within 3-5 mins of mixing trypan blue because lengthier incubation will mean cell death thus viability decreases

(f) A single drop of trypan blue/cell mixture allowed to trickle to hemocytometer. Now we use binocular microscope to focus on the cells in hemocytometer

(g)The viable & the non-viable cells are counted. The total no fo viable cell is obtained by multiplying by 2.The total cell count is given by adding viable and nonviable cell and multiplying by 2

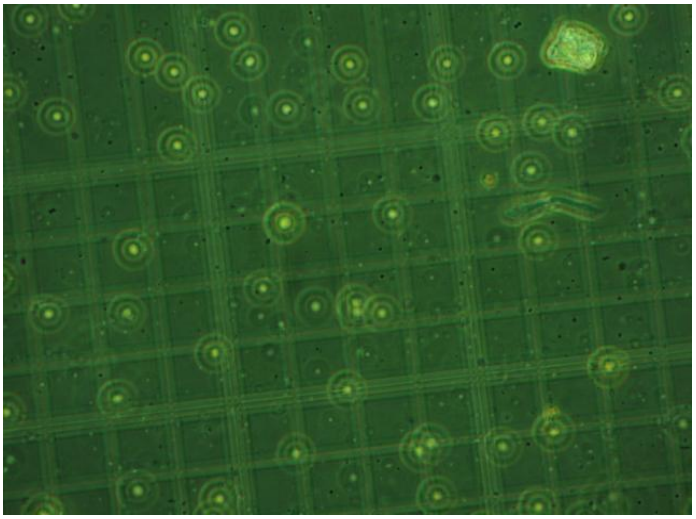
(h) The % of viable cell is calculated by $\frac{\text{total no of viable cell per ml of aliquot}}{\text{Total no cell per ml of aliquot}} \times 100$

.



Graph 4 the graph shows that maximum viability corresponds to a freezing rate of 1°C/min

Figure 9, 10 showing MNC after trypan blue staining under hemocytometer



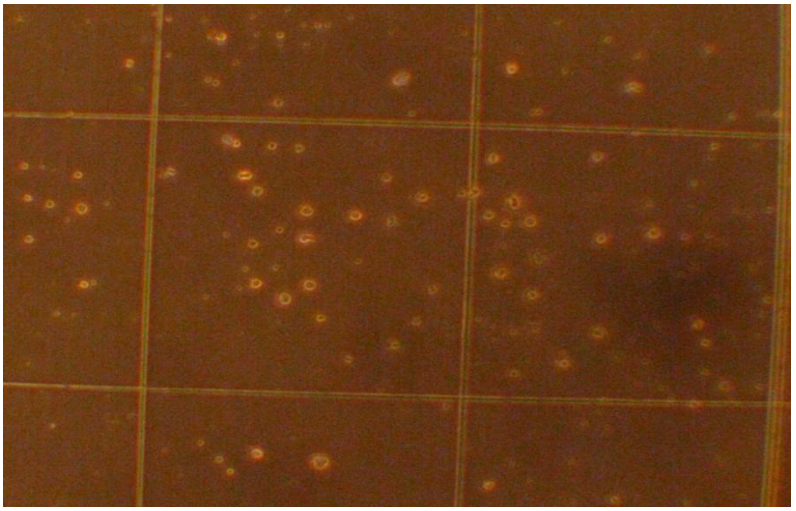
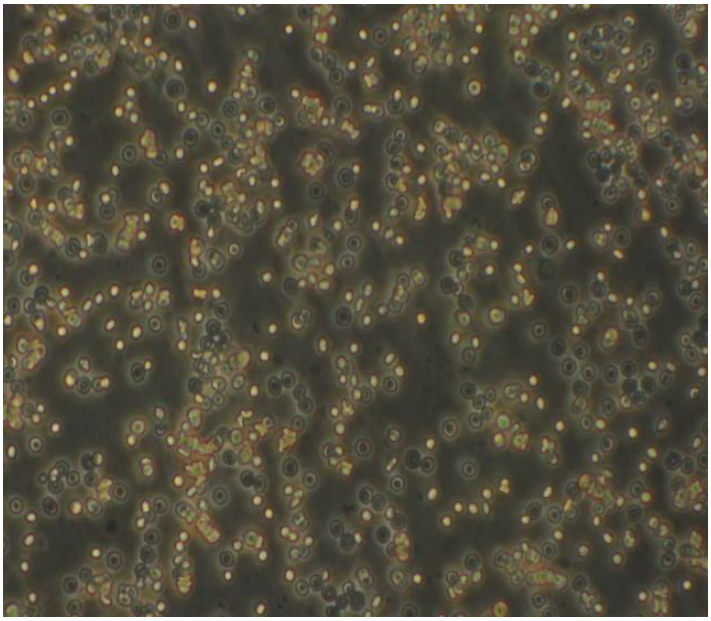


Figure 10, 11 showing MNC under phase contrast microscope



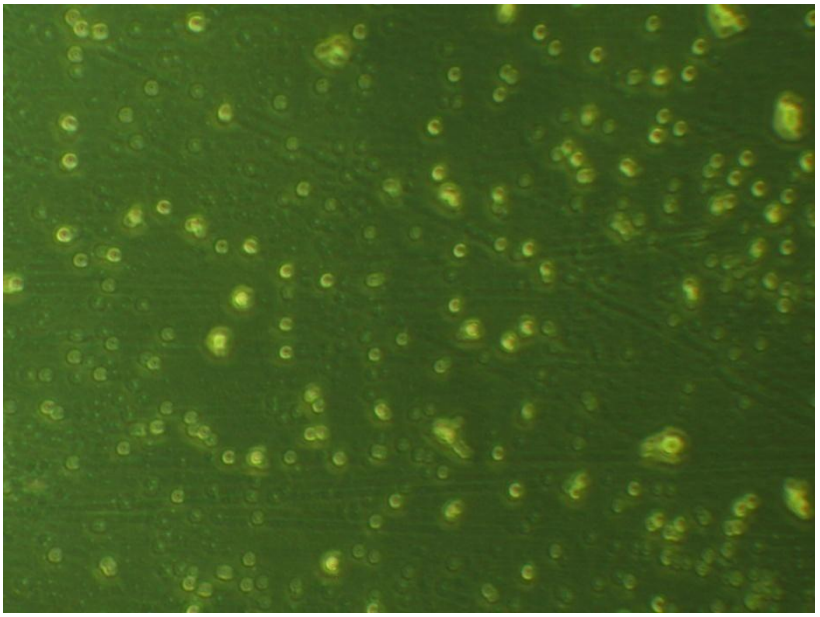
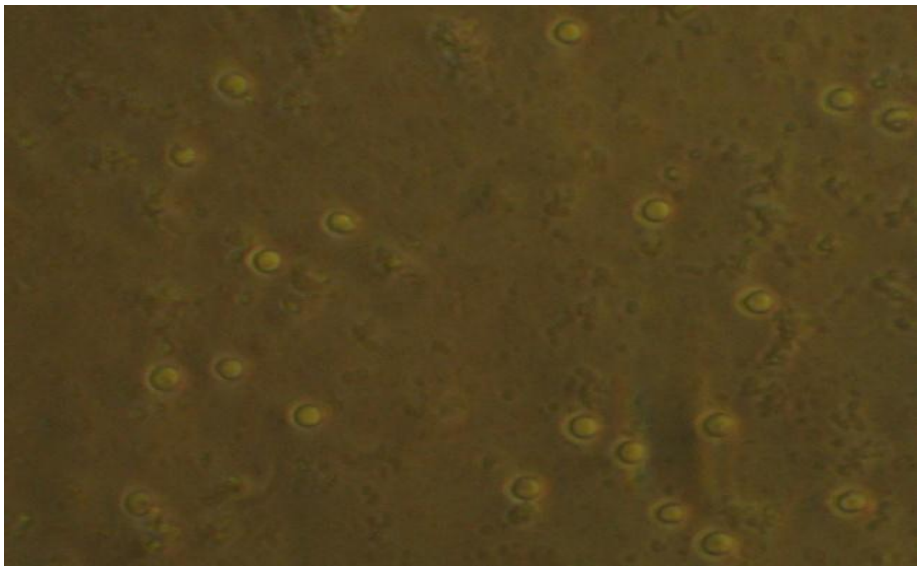
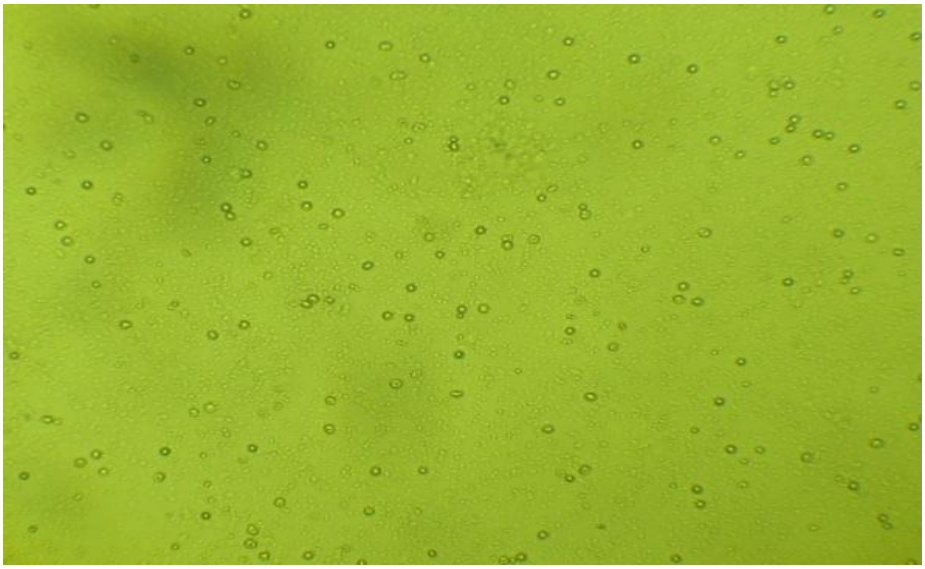


Figure 11 MNC's as seen in phase contrast microscope 20X

20X



10X



CHAPTER 3

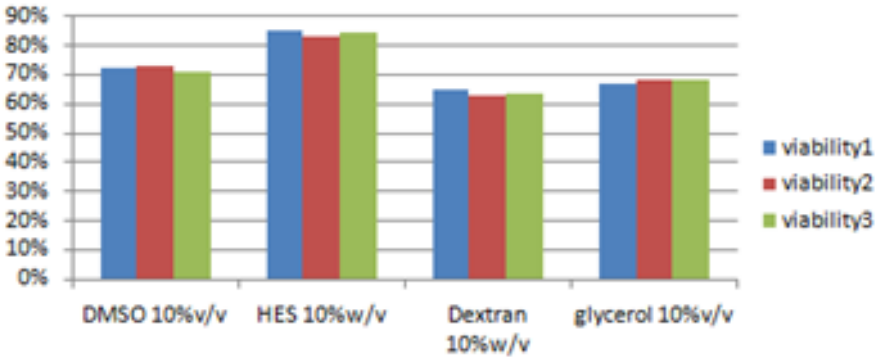
RESULTS AND DISCUSSION

3.1RESULTS

Table 5 showing viability of 3 batches of blood along with the corresponding cryoprotectant

Below that graph shows the viability in graphical way for three experimental re-runs

Cryoprotectants	viability1	viability2	viability3
DMSO 10%v/v	72%	73%	71.10%
HES 10%w/v	85%	83%	84.50%
Dextran 10%w/v	65%	63%	63.50%
glycerol 10%v/v	67%	68%	68.50%



Results obtained after freezing for one month is shown in tabular fashion. Hydroxy ethyl starch gives the best viability for MNC's while dextran the least and DMSO does an intermediate job after cryopreserved in liquid Nitrogen

The performance with dextran was dismal with 65%, 63%, 62.5%viability

From the experiment hydroxy ethyl starch emerges as the winner with viability 85%, 83%, and 84.5%

3.2 CONCLUSION

Mono nuclear cells, leukocyte or the police force of our body is vigilant 24X7 and fights off various diseases. Isolating MNC's would help us in treating various diseases as they are the spine of immune system .So their preservation is very important in emergency condition or when their count goes low as they for miniscule population of blood cells about four thousand to eleven thousand cells. Human whole blood and MNC's were successfully preserved through cryopreservation techniques. Hydroxy ethyl Starch should be the ideal choice of cryoprotectant for preserving MNC's as the viability achieved is around 85% in stark contrast to dextran which gives the lowest viability 65% which was found out under phase contrast using trypan blue protocol. This highlights the importance of cryoprotective agents which has an important role in suppressing intracellular and extracellular ice formation and also tells us that we can't just rely either on extracellular cryoprotectant or intracellular cryoprotectant we have to decided that from experimental observations .As mentioned earlier there is no standard protocol so for various preservation samples we have to find out experimentally using a host of cryoprotectant to find out which cryoprotectant gives the maximum viability. Hematopoietic stem cells can be isolated from peripheral blood which is preserved and can be used to produce their cell types. The RBC under SEM appears to be robust. MNC's preserved after thawing can be used for various immune assays. Controlled rate of freezing @ -1°C ensured the maximum viability which was is an important part of process optimization before dipping the vials in liquid nitrogen (-196°C).Thawing was also an important criteria to return the cell into their former pristine state which should be done very rapidly and I experienced it for real when I bungled up the thawing process with Hydroxy ethyl starch and then had to do it once again but was a liberating experience since the MNC viability was highest that is 85%.Human whole blood cryopreserved can come in very handy for anemic person, for erythrocyte disorders, for emergency purposes especially when there is a lack of rare blood group

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**

Introduction part is condensed and eclectically ccoalesced from various books, online sources and much of it is taken from(Day 2007) **Cryopreservation** and **freeze-drying** protocols so was the literature review to some extent

INSTRUMENTS USED FOR THE EXPERIMENTS

Lliquid nitrogen container

Controlled rate freezer

Phase contrast microscope

Laminar hood